JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Application of Bifidobacterial Phytases in Infant Cereals: Effect on Phytate Contents and Mineral Dialyzability

Juan Mario Sanz-Penella,[1,2] Carmen Frontela,[2] Gaspar Ros,[2] Carmen Martinez,[2] Vicente Monedero,[1] and Monika Haros*,[1]

[1]Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Av. Agustín Escardino 7, 46980 Paterna-Valencia, Spain

[2]Department of Food Science and Nutrition, Faculty of Veterinary Science and Food Science and Technology, University of Murcia, 30071 Murcia, Spain

ABSTRACT: Phytase activity was recently described in probiotic bifidobacterial strains, opening the possibilities for their use in foods, due to the generally regarded as safe/qualified presumption of safety status of these bacteria. Two raw materials for infant cereals (multicereal and gluten-free) were examined by measuring the *myo*-inositol phosphates content and the in vitro Ca, Fe, and Zn availability after a dephytinization process with purified phytases from *Bifidobacterium longum spp. infantis* and *Bifidobacterium pseudocatenulatum*. Treatment with both enzymes reduced the contents of phytate as compared to control samples (untreated or treated with fungal phytase) and led to increased levels of *myo*-inositol triphosphate. Dephytinization followed by an in vitro model of intestinal digestion increased the solubility of Zn. However, phytase treatment did not increase significantly the mineral dialyzability as compared to untreated samples. This is the first example of the application of purified bifidobacterial phytases in food processing and shows the potential of these enzymes to be used in products for human consumption.

KEYWORDS: *infant cereals, phytate-degrading enzymes, bifidobacterial phytase, phytate, myo-inositol phosphates, minerals*

INTRODUCTION

Phytate (*myo*-inositol hexakisphosphate or InsP_6) is a common constituent of plant foods such as cereals, oilseeds, legumes, or nuts, as well as roots, tubers, fruits, and vegetables. Many investigations have demonstrated that a diet rich in phytates may cause deficiencies in minerals, due to the formation of insoluble chelates.[1−5] The risk of these deficiencies is important mainly in animal nutrition or vulnerable population groups such as child-bearing women, strict vegetarians, inhabitants of developing countries, and also babies in the first years of life.[6,7] Studies in humans indicate that the absorption of iron,[2,8,9] calcium,[4,9] and zinc[4,10,11] from a meal could be impaired due to its phytate content, as it forms insoluble complexes with these minerals, thus compromising the health status of individuals.[3,12] This can be problematic in children from the fifth month of life onward. In this period, milk feeding is supplemented with other foods, such as cereals in the form of flours,[13] where mineral bioavailability is usually low because of the presence of phytates.[14] This problem worsens in developing countries, where infant cereals are in some cases taken after resuspension in water, instead of milk, with the concomitant reduction in minerals intake.[8]

Phytases are a group of enzymes that sequentially hydrolyze phytate, increasing the amount of available free phosphorus and decreasing phytate's affinity for different cations.[15] Cereals have an endogenous phytase; however, the amount of phytate in many products remains high due to inefficient enzymatic action in many processes.[16,17] The addition of exogenous phytase, mainly produced by microorganisms, appears to be the best strategy to effectively reduce InsP_6 in food and feed.[2,18−20] The use of phytases from microbial origins (*Schizosaccharomyces pombe, Penicillium funiculosum, Trichoderma reesei, Aspergillus niger, Aspergillus oryzae*, etc.) is currently approved for use in animal feed with regulations of the European Commision (EC) Nos. 785/2007, 1141/2007, 891/2010, 327/2010, and 171/2011 of the European Commission and aims to increase the availability of phosphorus from phytate, reducing its fecal excretion to the environment.[18] Conversely, although the use of exogenous phytases in human nutrition is currently not authorized, commercial phytases have been assayed in cereal products (bread) and legumes to reduce phytate content,[2,16,17,20−22] and interest exists in the screening of novel microbial phytases with improved characteristics for their use in food applications.[23] There are few reports dealing with their applications in infant cereals.[7,24] In this food matrix, phytase treatment generally resulted in enhanced mineral bioavailability, as reflected by the increased uptake of iron and zinc in intestinal epithelial (Caco-2) cell models.[24]

Haros et al.[25,26] found phytase activity in specific strains of the genus *Bifidobacterium*, suggesting its possible utility in food processing to significantly reduce the phytate content. Particularly, *Bifidobacterium longum* spp. *infantis* ATCC15697 and *Bifidobacterium pseudocatenulatum* ATCC27919 showed unique features in phytate hydrolytic profiles,[27] and their phytases have been recently cloned and characterized.[28] These strains have been used as starter cultures in whole wheat breads for developing bakery products with low InsP_6 content[29,30] and increased iron accessibility.[31] The bifidobacteria are considered GRAS/QPS (generally regarded as safe/qualified presumption

Received: August 3, 2012
Revised: November 5, 2012
Accepted: November 6, 2012
Published: November 14, 2012

dx.doi.org/10.1021/jf3034013 | *J. Agric. Food Chem.* 2012, 60, 11787−11792

of safety) microorganisms, making this a promising strategy to reduce phytate levels in processed products intended for human consumption.

The main goal of the present investigation was to determine the extent to which the phytases isolated from *B. longum* spp. *infantis* and *B. pseudocatenulatum* modify the *myo*-inositol phosphates composition during infant cereals production [multicereal (MC) and gluten-free (GF)] and their effects on the solubility and dialyzability of minerals.

MATERIALS AND METHODS

Materials and Chemicals. The material used in the current investigation was the staple for preparation of commercial infant cereals, and it was provided by Hero España S.A. (Alcantarilla, Spain). Two formulations were studied, MC (containing rice, oat, barley, rye, maize, millet, sorghum, and wheat) and GF cereals (containing rice, maize, and tapioca).

Digestive enzymes and bile salts were supplied by Sigma-Aldrich (St. Louis, MO): pepsin (porcine, catalogue no. P-7000), pancreatin (porcine, catalogue no. P-1750), and bile extract (porcine, catalogue no. B-8756). The enzymes solutions used for the in vitro simulation of digestion in infants were prepared according to Frontela et al.[14] All other chemicals were of the analytical grade commercially available. Commercial fungal phytase was from *A. oryzae* (EC 3.1.3.26, Stern-Enzym GmbH & Co. KG, Ahrensburg, Germany).

Purification of Bifidobacterial Phytases. The bifidobacterial phytases were expressed as 6xHis-tagged proteins in *Escherichia coli* and purified as described.[28] Briefly, *E. coli* M15 clones carrying the expression vectors were grown in 1 L of LB medium with 100 μg ampicillin per mL at 37 °C, and induction was started by addition of isopropyl-β-D-thiogalactopyranoside to 0.1 mM when the cultures reached an OD550 nm of 0.4. Growth continued for 4 h at 30 °C, and the bacterial cells were recovered by centrifugation, washed with 0.9% NaCl, and resuspended in 100 mM Tris-HCl buffer, pH 7.4, supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithioerythritol, and 1 mg/mL lysozyme. After 30 min at 37 °C, the cells were sonicated (five pulses of 20 s) and afterward frozen with liquid nitrogen. After they were defreezed, the cytoplasmic content was recovered by centrifugation (17000*g*, 30 min, 4 °C in a Sorvall SS34 rotor), and the supernatants were filtered through 0.45 μm pore size filters. The filtered lysates were transferred to 1 mL of Ni-NTA columns (Qiagen, Valencia, United States) previously equilibrated with 50 mM Tris-HCl, 10% glycerol, and 50 mM Na_2SO_4 (buffer A). After two washing steps with 20 mL of buffer A and 20 mL of buffer A containing 30 mM imidazole, proteins were eluted with buffer A plus 300 mM imidazole. One milliliter fractions were recovered and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1). Fractions containing the expressed proteins were dialyzed against 100 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, and 50 mM NaCl and stored at −80 °C.

Infant Cereal Process at Laboratory Scale. The usual steps in the industrial production of infant cereals include cereal mixing, roasting, α-amylase treatment, drying, and fortification (Figure 2). The *Bifidobacterium* phytases (or commercial fungal phytase as positive control) were included during the dextrinization step together with α-amylase. The phytase dose utilized was 20-fold that of the endogenous phytase activity from the cereal mix, measured under the same conditions (0.95 U/g flour for MC and 0.88 U/g flour for GF). Samples were processed as follows: first, each infant cereal mix was roasted in an oven (P-Selecta, model 201, Barcelona, Spain) at 120 °C for 30 min. Then, 60 g of roasted flour was suspended in 124 mL of deionized water, and 700 mg per kg of flour of α-amylase (EC. 3.2.1.1, from human saliva, Sigma Chemical Co., St. Louis, MO) together with the different phytases were added. The pH was adjusted to 5.5, and the mixtures were incubated at 55 °C with gentle agitation for 20 min. The reaction was stopped by heating the slurry at 98 °C for 10 min. The mixtures were dried at 120 °C in a drying cabinet and milled in a grinder (Super Junior "S", 60 g, Ecully Cedex, France) to reduce the particle size. Finally, infant cereals were fortified with iron [elemental iron, 0.008% (w/w)] and calcium [dicalcium phosphate, 1.1% (w/w)] according to manufacturer's recommendations.

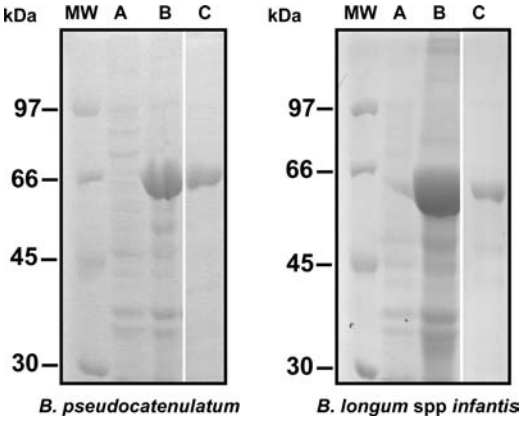

Figure 1. SDS-PAGE analysis of purified phytases from *B. pseudocatenulatum* and *B. longum* spp. *infantis*. MW, molecular weight marker; A, uninduced *E. coli* extracts; B, induced *E. coli* extracts; and C, purified 6x(His) enzymes. Numbers are molecular weights in kDa.

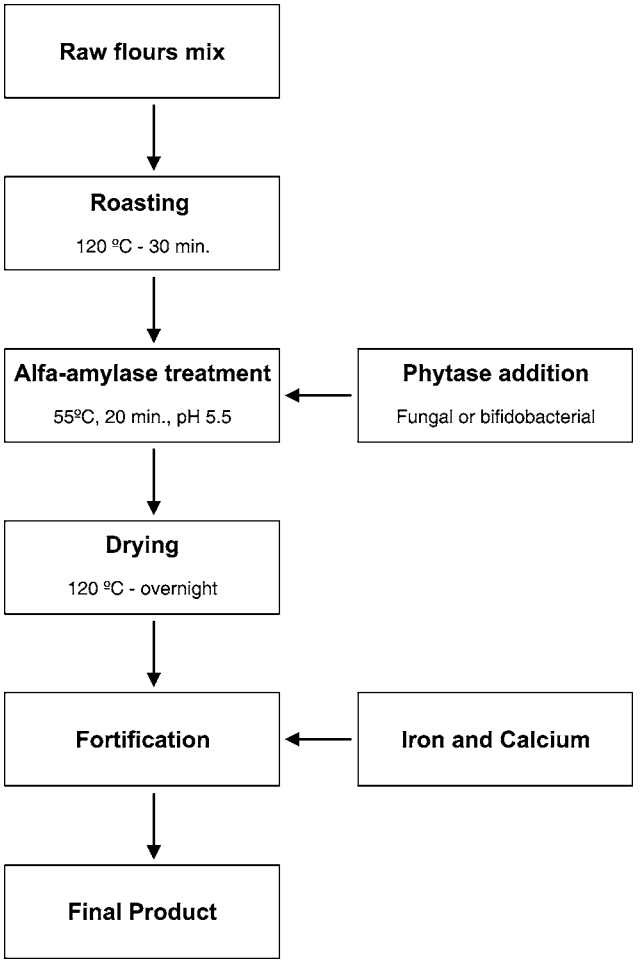

Figure 2. Flowsheet of infant cereal preparation at laboratory scale.

Determination of Phytase Activity. The phytase activity was spectrophotometrically determined by measuring the amount of liberated inorganic phosphate from potassium phytate (Sigma-Aldrich) by a colorimetric technique using molybdovanadate[32] according to Haros et al.[21,25] One unit (U) was defined as the amount of enzyme

Table 1. *myo*-Inositol Phosphates, Mineral Contents, and Molar Ratios between InsP_6 and Minerals on MC Samples[a]

parameter	units	raw material	control sample	phytase		
				fungal	*B. longum*	*B. pseudocatenulatum*
			myo-inositol phosphates			
InsP_6	μmol/g DM	2.32 d	1.16 c	0.38 b	0.23 ab	0.15 a
InsP_5	μmol/g DM	1.77 c	1.41 c	0.87 b	0.65 ab	0.38 a
InsP_4	μmol/g DM	0.49 a	1.84 d	1.12 c	0.70 b	0.48 a
InsP_3	μmol/g DM	0.15 a	0.77 b	1.54 c	2.39 d	1.80 c
InsP_6 + InsP_5	μmol/g DM	4.08 d	2.58 c	1.25 b	0.88 ab	0.54 a
			minerals			
Ca	mg/100 g DM	ND	171.00 a	201.00 a	189.00 a	205.00 a
Fe	mg/100 g DM	ND	6.58 a	6.41 a	6.04 a	6.49 a
Zn	mg/100 g DM	ND	0.61 a	0.74 a	0.61 a	0.88 a
			molar ratios[b]			
InsP_6/Ca > 0.24	mol/mol	ND	0.02 a	0.01 a	0.00 a	0.00 a
InsP_6/Fe > 1	mol/mol	ND	0.85 c	0.28 b	0.17 ab	0.11 a
InsP_6/Zn > 5	mol/mol	ND	6.12 c	2.01 b	1.24 ab	0.82 a
InsP_6 × Ca/Zn > 150−200	mol/mol	ND	301.41 c	98.95 b	60.40 a	39.92 a

[a]DM, dry matter; InsP_3 to InsP_6: *myo*-inositol containing 3−6 phosphates per inositol residue; ND, not determined. Mean, $n = 3$, values followed by the same letter in the same row are not significantly different at the 95% confidence level. [b]Predictive value of low mineral bioavailability.[46−48].

Table 2. *myo*-Inositol Phosphates, Mineral Contents, and Molar Ratios between InsP_6 and Minerals on GF Samples[a]

parameter	units	raw material	control sample	phytase		
				fungal	*B. longum*	*B. pseudocatenulatum*
			myo-inositol phosphates			
InsP_6	μmol/g DM	1.93 d	1.10 c	0.61 b	0.27 a	0.16 a
InsP_5	μmol/g DM	1.57 e	1.28 d	0.95 c	0.74 b	0.48 a
InsP_4	μmol/g DM	0.49 ab	1.28 d	0.66 bc	0.71 c	0.39 a
InsP_3	μmol/g DM	0.04 a	0.57 b	0.83 c	1.17 d	0.52 b
InsP_6+InsP_5	μmol/g DM	3.50 e	2.38 d	1.55 c	1.00 b	0.64 a
			minerals			
Ca	mg/g DM	ND	200.00 a	180.00 a	200.00 a	180.00 a
Fe	mg/g DM	ND	6.20 a	6.32 a	5.74 a	6.75 a
Zn	mg/g DM	ND	1.15 a	1.20 a	1.18 a	0.94 a
			molar ratios[b]			
InsP_6/Ca > 0.24	mol/mol	ND	0.02 b	0.01 a	0.01 a	0.00 a
InsP_6/Fe > 1	mol/mol	ND	0.79 c	0.45 b	0.20 a	0.12 a
InsP_6/Zn > 5	mol/mol	ND	6.11 c	3.44 b	1.51 a	0.92 a
InsP_6 × Ca/Zn > 150−200	mol/mol	ND	299.63 c	165.38 b	72.98 a	44.56 a

[a]DM, dry matter; InsP_3 to InsP_6: *myo*-inositol containing 3−6 phosphates per inositol residue; ND, not determined. Mean, $n = 3$, values followed by the same letter in the same row are not significantly different at the 95% confidence level. [b]Predictive value of low mineral bioavailability.[46−48].

releasing 1 μg of inorganic phosphate per minute at 50 °C and pH 5.5 (100 mM sodium acetate buffer).

Determination of *myo*-Inositol Phosphates. The initial InsP_6 concentration in cereals, the remaining concentration of InsP_6 in processed cereals, and the lower *myo*-inositol phosphates generated were measured by high-pressure liquid chromatographic method described by Türk and Sandberg,[20] later modified by Sanz-Penella et al.[22] Identification of the *myo*-inositol phosphates was achieved by comparison with standards of phytic acid dipotassium salt (Sigma-Aldrich). Samples were analyzed in triplicate.

In Vitro Digestion. The solubility and dialysis of iron, calcium, and zinc in infant cereals were determined by the in vitro method described by Miller et al.[33] with suitable modifications adapted to the gastrointestinal conditions of infants younger than 6 months.[7]

Mineral Content of Cereals. The total Fe, Ca, and Zn concentrations in soluble and dialyzed fractions were determined using a flame atomic absorption spectrometer.[7] For mineral determination, the glass material was washed with detergent, soaked in concentrated nitric acid (1.41 g/mL), and rinsed three times with deionized water before use.

Statistical Analysis. Multiple sample comparison was statistically analyzed with the Statgraphics Plus 5.0. Fisher's least significance difference (LSD) test was used to compare means at the 5% significance level.

RESULTS AND DISCUSSION

Degradation of Phytate and Generation of Lower *myo*-Inositol Phosphates in Infant Cereals. Because of the action of the phytase enzymes, InsP_6 is sequentially hydrolyzed to a mixture of *myo*-inositol phosphates with 5, 4, 3, 2, and 1 phosphate groups in their structures (InsP_5, InsP_4, InsP_3, InsP_2, and InsP_1, respectively). The effect of phytase treatment during infant cereal preparation process on the *myo*-inositol phosphates composition is summarized in Tables 1 and 2. The amounts of phytates found in infant flours were 1.93 and 2.32 μmol/g for GF and MC samples, respectively. During the infant cereal process, endogenous phytate-degrading enzymes (phytases and/or unspecific phosphatases) from cereals[34,35] were active as denoted by the phytate hydrolysis in control

samples as compared with the raw materials. The inclusion of exogenous phytases had an additional and significant reduction of phytate levels between 68.6 to 93.4%, as compared to phytate levels in raw materials (Figure 3). The addition of

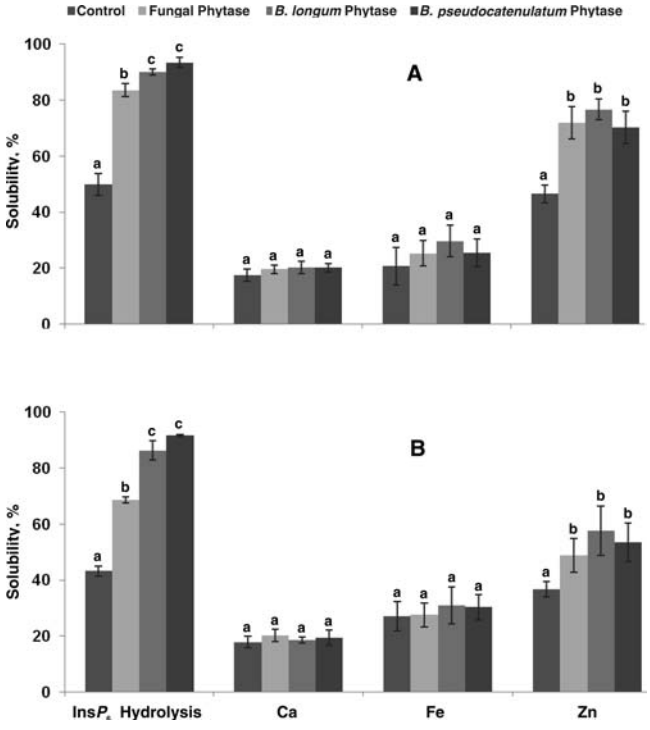

Figure 3. Effect of phytases on phytate hydrolysis and mineral solubility. (A) MC sample. (B) GF sample.

Bifidobacterium phytases, purified after heterologous expression in *E. coli* (Figure 1), resulted in a decrease in the amount of InsP_6 between 7- and 15-fold as compared to its initial concentration in raw materials and between 5- and 7.7-fold as compared to untreated control samples. A previous research studied the effects of fungal phytase addition to different types of commercial infant cereals slurries.[7] In the research reported here, the whole industrial process was reproduced (Figure 2), showing that the dephytinization step can be performed together with the dextrinization step, leading to an efficient hydrolysis of phytate. The results were compared with cereals treated with a commercial fungal (*A. oryzae*) phytase as positive control, showing that this enzyme was less effective in hydrolyzing InsP_6 than the bifidobacterial enzymes under our experimental conditions. The lower effect of the fungal phytase as compared to the bacterial phytases is probably not caused by differences in their optimal pH, a key factor in phytase activity[36] (optimum pH for the phytase from *A. oryzae* has been reported to be in the range of 4.5−5.5,[37,38] while the optimum pH for bifidobacterial phytases was 5.5, retaining virtually 100% of the activity between 5 and 6[28]), because the same units of the different enzymes, determined at pH 5.5 and 50 °C, were added during the process. Therefore, the differences in phytate hydrolysis may rely on the presence of inhibitory substances, different stabilities, or different activities on specific *myo*-inositol phosphates, which may affect enzyme performance in the cereal slurry. Moreover, the optimal temperature for *A. oryzae* phytases is between 40 and 50 °C,[37] which is lower than the temperature applied in the process (55 °C). On the other hand, the optimum temperature of bacterial phytases is 50 °C for *B. longum* spp. *infantis* and 50−55 °C for *B. pseudocatenulatum*, being both extremely specific for phytate.[28] The inclusion of phytases also reduced significantly the *myo*-inositol pentakisphosphate (InsP_5) concentrations as compared to control samples (Tables 1 and 2). As occurred with InsP_6, phytases from bifidobacteria were more effective than the fungal phytase in hydrolyzing InsP_5. This result could have important consequences in mineral bioavailability because, similar to InsP_6, InsP_5 also acts as a strong chelator, exerting negative effects on mineral solubility.[30,39] Because of endogenous and/or exogenous enzymatic activity on InsP_6 and InsP_5, the concentration of InsP_4 and InsP_3 were significantly higher in phytase-treated samples than in raw materials. Accumulation of InsP_3 was higher in samples treated with the bifidobacterial enzymes (except for *B. pseudocatenulatum* in GF samples), although differences were only significant for the *B. longum* spp. *infantis* phytase. This is in agreement with the *myo*-inositol phosphate products generated from phytate by both bifidobacterial strains and their purified enzymes, which showed accumulation of InsP_3, which was not further hydrolyzed to lower *myo*-inositol phosphates.[26−28] The different profile of lower *myo*-inositol phosphates generated by distinct phytases may have important health implications, as many biological functions have been linked to *myo*-inositol phosphates.[40,41] For example, it has been suggested that some InsP_3 isomers, especially the 1,2,3-InsP_3 isomer, play a role in iron chelation as an intracellular iron transport agent and/or cellular antioxidant.[42] Therefore, dephytinization could not only positively affect human health by increasing the bioavailability of minerals but also as a result of synthesis of potentially bioactive inositol-phosphates.

Effect of Dephytinization on Mineral Availability. We investigated how phytase treatment influenced mineral availability by measuring iron, calcium, and zinc solubility and dialyzability after the samples were subject to a treatment mimicking gastrointestinal conditions in infants. Phytase addition to MC infant flour did not increase significantly the solubility of iron and calcium (Figure 3). However, concerning the effects of dephytinization on zinc solubility, this was increased by 25−30%, showing no significant differences between phytases (Figure 3). In general, dephytinization did not affect mineral dialyzability. For calcium and zinc, the values ranged from 4.6 to 10.4%, whereas iron dialyzability reached values up to 0.7%. The poor dialyzability percentage observed for iron could probably be explained by the source of enrichment used, elemental iron.[43] These results are in agreement with other researches, which evidenced no improvement of iron, calcium, and zinc dialyzability by commercial phytase addition to different food matrices.[19,44] However, recent research demonstrated that fermentation of whole wheat bread with phytase-producing bifidobacteria increased iron dialyzability, although this did not result in increased iron uptake in a Caco-2 cells model.[31] A similar situation was reported for the dephytinization of fava bean flour, which enhanced iron solubility.[45]

The marked inhibitory effect of small amounts of phytate (≥0.135 μmol/g) on iron bioavailability in fortified bread pretreated with phytases has been demonstrated.[2] In this regard, some critical values of the phytate/minerals molar ratios have been defined. A phytate/Fe molar ratio >1 resulted in low iron bioavailability, with preferable values situated below 0.4.[46] Also, a phytate/Ca molar ratio >0.24 would impair calcium bioavailability,[47] whereas the zinc absorption could be less than

50% when the phytate/Zn molar ratio is >5 [41]or could be affected by phytate × Ca/Zn molar ratio above 150−200.[48] In MC infant flours, dephytinization did not improve significantly iron and calcium solubility. The InsP_6/Ca or InsP_6/Fe molar ratios were below the critical threshold in control samples (without phytase addition). This was due to the fortification process, which increased calcium and iron concentrations. Therefore, the hydrolysis of phytate would not have an effect on these minerals if samples are fortified (Table 1). In different cereal products (i.e., whole wheat breads), InsP_6/Ca or InsP_6/Fe molar ratios follow the opposite tendency, evidencing a deep inhibition of mineral availability.[30] In the case of zinc, the phytate/Zn molar ratio of control sample (6.1) was found higher than 5, which would compromise mineral availability. The addition of phytases to the infant cereal process reduced both the phytate/Zn and the phytate × Ca/Zn molar ratios below 5 and 150, respectively, which would have a positive impact on zinc bioavailability.

Similar to MC samples, the treatment of GF cereal samples with either commercial phytase or phytases from bifidobacteria did not result in higher calcium or iron solubility with respect to control samples (Figure 3). Nevertheless, the solubility of zinc was significantly increased by 17 and 21% with the addition of phytases from *B. longum* spp. *infantis* and *B. pseudocatenulatum*, respectively. Regarding the dialyzability, no significant differences were observed among samples. Whereas dialyzability did not exceed 1% for iron, the values were found between 4.0 and 5.4% for zinc and from 9.1 to 11.1% for calcium. The InsP_6/Ca or InsP_6/Fe ratios were below the critical values, which would not compromise mineral availability (Table 2). As in the MC samples, an improvement in solubility/dialyzability of iron and calcium by addition of phytase was not observed. However, phytase treatment reduced phytate/Zn and phytate × Ca/Zn molar ratios from 6.1 to 0.9 and from 300 to 45, respectively, indicating an enhancement on mineral availability (Table 2). The increase in zinc solubility percentages after phytase treatment obtained here was higher that those reported for other infant cereal mixes after a fungal phytase treatment.[7] However, in that assay, the type of sample reconstitution (water or follow-on milk formula) was the main factor affecting mineral availability. Also, and as previously reported,[7] solubility did not follow the same trend as dializability, probably due to the complexation of minerals with high molecular weight components above the molecular weight cut off of the utilized dialysis membranes (12000 Da).

In summary, this is the first time that purified bifidobacterial phytases are included in a food production process. This resulted in an efficient phytate hydrolysis during infant cereals production, which rendered lower *myo*-inositol phosphate products, which may possess specific functional roles. Bifidobacterial phytase treatment increased zinc solubility, although calcium and iron solubility did not change and mineral dialyzability also remained unchanged. Bifidobacteria are intestinal GRAS/QPS microorganisms with probiotic characteristics. This research evidenced the possibilities of the use of their phytases in products for human consumption. New research is under way to test their efficacy in different phytate-rich food products.

AUTHOR INFORMATION

Corresponding Author

*Tel: +34 96 390 00 22. Fax: +34 96 363 63 01. E-mail: mharos@iata.csic.es.

Funding

This work was financially supported by funds of the Consolider Fun-C-Food project CSD2007-00063 from the Ministry of Economy and Competitiveness and PROMETEO/2012/064 from Generalitat Valenciana, from Spain. J.M.S.-P. was the recipient of a predoctoral fellowship from the Ministry of Economy and Competitiveness from Spain. We thank Hero España S.A. for providing the cereal samples.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ATCC, American Type Culture Collection; DM, dry matter; EC, enzyme commission; EDTA, ethylenediaminetetraacetic acid; GF, gluten-free; GRAS/QPS, generally regarded as safe/qualified presumption of safety; InsP_6, phytic acid, *myo*-inositol hexakisphosphate or phytate; InsP_5, *myo*-inositol pentakisphosphate; InsP_4, *myo*-inositol tetrakisphosphate; InsP_3, *myo*-inositol triphosphate; InsP_2, *myo*-inositol diphosphate; InsP_1, *myo*-inositol monophosphate; MC, multicereal; OD, optical density; PU, phytase units; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis

REFERENCES

(1) Turnlund, J. R.; King, J. C.; Keyes, W. R.; Gong, B.; Michel, M. C. A stable isotope study of zinc absorption in young men: Effects on phytate and α-cellulose. *Am. J. Clin. Nutr.* **1984**, *40*, 1071−1077.

(2) Sandberg, A. S.; Hulthen, L. R.; Türk, M. Dietary *Aspergillus niger* phytase increases iron absorption in humans. *J. Nutr.* **1996**, *126*, 476−480.

(3) Konietzny, U.; Greiner, R. Phytic acid: Properties and Determination. In *Encyclopaedia of Food Science and Nutrition*, 2nd ed.; Caballero, B., Trugo, L., Finglas, P., Eds.; Elsevier: London, United Kingdom, 2003; pp 4555−4563.

(4) Fredlund, K.; Isaksson, M.; Rossander-Hulthén, L.; Almgren, A.; Sandberg, A. S. Absorption of zinc and retention of calcium: Dose-dependent inhibition by phytate. *J. Trace Elem. Med. Biol.* **2006**, *20*, 49−57.

(5) Bohn, L.; Meyer, A. S.; Rasmussen, S. K. Phytate: Impact on environment and human nutrition. A challenge for molecular breeding. *J. Zhejiang Univ. Sci. B* **2008**, *9*, 165−191.

(6) Rimbach, G.; Pallauf, J.; Moehring, J.; Kraemer, K.; Minihane, A. M. Effect of dietary phytate and microbial phytase on mineral and trace element bioavailability - a literature review. *Curr. Top. Nutraceutical Res.* **2008**, *6*, 131−144.

(7) Frontela, C.; Haro, J. F.; Ros, G.; Martinez, C. Effect of dephytinization and follow-on formula addition on *in vitro* iron, calcium, and zinc availability from infant cereals. *J. Agric. Food Chem.* **2008**, *56*, 3805−3811.

(8) Hurrell, R. F.; Reddy, M. B.; Juillerat, M. A.; Cook, J. D. Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. *Am. J. Clin. Nutr.* **2003**, *77*, 1213−1219.

(9) Ma, G.; Li, Y.; Jin, Y.; Zhai, F.; Kok, F. J.; Yang, X. Phytate intake and molar ratios of phytate to zinc-iron and calcium in the diets of people in China. *Eur. J. Clin. Nutr.* **2007**, *61*, 368−374.

(10) Wise, A. Phytate and zinc bioavailability. *Int. J. Food Sci. Nutr.* **1995**, *46*, 53−63.

(11) Krebs, N. F. Food choices to meet nutritional needs of breast-fed infants and toddlers on a mixed diet. *J. Nutr.* **2007**, *137*, 511−517.

(12) Lopez, H. W.; Leenhardt, F.; Coudray, C.; Remesy, C. Minerals and phytic acid interactions: Is it a real problem for human nutrition? *Int. J. Food Sci. Technol.* **2002**, *37*, 727−739.

(13) Kersting, M.; Dulon, M. Assessment of breast-feeding promotion in hospitals and follow-up survey of mother-infant pairs in Germany: The SuSe Study. *Public Health Nutr.* **2002**, *5*, 547−552.

(14) Frontela, C.; García-Alonso, F. J.; Ros, G.; Martínez, C. Phytic acid and inositol phosphates in raw flours and infant cereals: The effect of processing. *J. Food Compos. Anal.* **2008**, *21*, 343−350.
(15) Vohra, A.; Satyanarayana, T. Phytases: Microbial sources, production, purification, and potential biotechnological applications. *Crit. Rev. Biotechnol.* **2003**, *23*, 29−60.
(16) Greiner, R.; Konietzny, U.; Jany, K. D. Purification and properties of a phytase from rye. *J. Food Biochem.* **1998**, *22*, 143−161.
(17) Haros, M.; Rosell, C. M.; Benedito, C. Fungal phytase as a potencial breadmaking additive. *Eur. Food Res. Technol.* **2001**, *213*, 317−322.
(18) Maenz, D. D. Enzymatic characteristics of phytases as they relate to their use in animal feeds. In *Enzymes in Farm Animal Nutrition*; Bedford, M. R., Partridge, G. G., Eds.; CABI Publishing: New York, NY, 2001; pp 61−84.
(19) Porres, J. M.; Etcheverry, P.; Miller, D. D.; Lei, X. G. Phytase and citric acid supplementation in whole-wheat bread improves phytate-phosphorus release and iron dialyzability. *J. Food Sci.* **2001**, *66*, 614−619.
(20) Türk, M.; Sandberg, A. S. Phytate degradation during breadmaking—Effect of phytase addition. *J. Cereal Sci.* **1992**, *15*, 281−294.
(21) Haros, M.; Rosell, C. M.; Benedito, C. Use of fungal phytase to improve breadmaking performance of whole wheat bread. *J. Agric. Food Chem.* **2001**, *49*, 5450−5454.
(22) Sanz-Penella, J. M.; Collar, C.; Haros, M. Effect of wheat bran and enzyme addition on dough functional performance and phytic acid levels in bread. *J. Cereal Sci.* **2008**, *48*, 715−721.
(23) Shim, J.-H.; Oh, B.-C. Characterization and application of calcium-dependent β-Propeller phytase from *Bacillus amyloliquefaciens* DS11. *J. Agric. Food Chem.* **2012**, *60*, 7532−7537.
(24) Frontela, C.; Scarino, M. L.; Ferruzza, S.; Ros, G.; Martínez, C. Effect of dephytinization on bioavailability of iron, calcium and zinc from infant cereals assessed in the Caco-2 cell model. *World J. Gastroenterol.* **2009**, *15*, 1977−1984.
(25) Haros, M.; Bielecka, M.; Sanz, Y. Phytase activity as a novel metabolic feature in *Bifidobacterium*. *FEMS Microbiol. Lett.* **2005**, *247*, 231−239.
(26) Haros, M.; Bielecka, M.; Honke, J.; Sanz, Y. *Myo*-inositol hexakisphosphate degradation by *Bifidobacterium infantis*. *Int. J. Food Microbiol.* **2007**, *117*, 76−84.
(27) Haros, M.; Carlsson, N. G.; Almgren, A.; Larsson-Alminger, M.; Sandberg, A. S.; Andlid, T. Phytate degradation by human gut isolated *Bifidobacterium pseudocatenulatum* ATCC27919 and its probiotic potential. *Int. J. Food Microbiol.* **2009**, *135*, 7−14.
(28) Tamayo-Ramos, J. A.; Sanz-Penella, J. M.; Yebra, M. J.; Monedero, V.; Haros, M. Novel phytases from *Bifidobacterium pseudocatenulatum* ATCC 27919 and *Bifidobacterium longum* spp *infantis* ATCC 15697. *Appl. Environ. Microbiol.* **2012**, *78*, 5013−5015.
(29) Sanz-Penella, J. M.; Tamayo-Ramos, J. A.; Sanz, Y.; Haros, M. Phytate reduction in bran-enriched bread by phytase-producing Bifidobacteria. *J. Agric. Food Chem.* **2009**, *57*, 10239−10244.
(30) Sanz-Penella, J. M.; Tamayo-Ramos, J. A.; Haros, M. Application of bifidobacteria as starter culture in whole wheat sourdough breadmaking. *Food Bioprocess Technol.* **2012**, *5*, 2370−2380.
(31) Sanz-Penella, J. M.; Laparra, J. M.; Sanz, Y.; Haros, M. Assessment of iron bioavailability in whole wheat bread by addition of phytase-producing bifidobacteria. *J. Agric. Food Chem.* **2012**, *60*, 3190−3195.
(32) Tanner, J. T.; Barnett, S. A. Methods of analysis for infant formula: Food and drug administration and infant formula Council Collaborative study, Phase III. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 777−785.
(33) Miller, D. D.; Schricker, B. R.; Rasmussen, R. R.; Van Campen, D. An in vitro method for estimation of iron bioavailability from meal. *Am. J. Clin. Nutr.* **1981**, *34*, 2248−2256.
(34) Oomah, B. D.; Luc, G.; Leprelle, C.; Drover, J. C. G.; Harrison, J. E.; Olson, M. Phenolics, phytic acid, and phytase in Canadian-grown low-tannin faba bean (*Vicia faba* L.) genotypes. *J. Agric. Food Chem.* **2011**, *59*, 3763−3771.
(35) Dai, F.; Qiu, L.; Xu, Y.; Cai, S.; Qiu, B.; Zhang, G. Differences in phytase activity and phytic acid content between Cultivated and Tibetan annual wild barleys. *J. Agric. Food Chem.* **2010**, *58*, 11821−11824.
(36) Leenhardt, F.; Levrat-Verny, M. A.; Chanliaud, E.; Remesy, C. Moderate decrease of pH by sourdough fermentation is sufficient to reduce phytate content of whole wheat flour through endogenous phytase activity. *J. Agric. Food Chem.* **2005**, *53*, 98−102.
(37) Fujita, J.; Yamane, Y. I.; Fukuda, H.; Kizaki, Y.; Wakabayashi, S.; Shigeta, S.; Suzuki, O.; Ono, K. Production and properties of phytase and acid phosphatase from a sake koji mold *Aspergillus oryzae*. *J. Biosci. Bioeng.* **2003**, *95*, 348−353.
(38) Uchida, H.; Arakida, S.; Sakamoto, T.; Kawasaki, H. Expression of *Aspergillus oryzae* phytase gene in *Aspergillus oryzae* RIB40 niaD(-). *J. Biosci. Bioeng.* **2006**, *102*, 564−567.
(39) Sandberg, A. S.; Brune, M.; Carlsson, N. G.; Harrberg, L.; Skoglund, E.; Rossander-Hulthen, L. Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *Am. J. Clin. Nutr.* **1999**, *70*, 240−246.
(40) Shi, Y.; Azab, A. N.; Thompson, M. N.; Greenberg, M. L. Inositol phosphates and phosphoinositides in health and disease. *Sub-Cell. Biochem.* **2006**, *39*, 265−292.
(41) Suzuki, T.; Nishioka, T.; Ishizuka, S.; Hara, H. A novel mechanism underlying phytate-mediated biological action-phytate hydrolysates induce intracellular calcium signalling by Gαq protein-coupled receptor and phospholipase C-dependent mechanism in colorectal cancer cells. *Mol. Nutr. Food Res.* **2010**, *54*, 947−955.
(42) Veiga, N.; Torres, J.; Mansell, D.; Freeman, S.; Dominguez, S.; Barker, C. J.; Diaz, A.; Kremer, C. Chelatable iron pool: inositol 1,2,3-trisphosphate fulfils the conditions required to be a safe cellular iron ligand. *J. Biol. Inorg. Chem.* **2009**, *14*, 51−59.
(43) Kapsokefalou, M.; Alexandropoulou, I.; Komaitis, M.; Politis, I. *In vitro* evaluation on iron solubility and dialyzability of various iron fortificants and of iron-fortified milk products targeted for infants and toddlers. *Int. J. Food Sci. Nutr.* **2005**, *56*, 293−302.
(44) Dyner, L.; Drago, S. R.; Piñeiro, A.; Sánchez, H.; González, R.; Villamil, E.; Valencia, M. E. Composition and potential contribution of iron, calcium and zinc of bread and pasta made with wheat and amaranth flours. *Arch. Latinoam. Nutr.* **2007**, *57*, 69−78.
(45) Luo, Y.-W.; Xie, W.-H.; Cui, Q.-X. Effects of phytase, cellulase, and dehulling treatments on iron and zinc *in vitro* solubility in Faba Bean (*Vicia faba* L.) flour and legume fractions. *J. Agric. Food Chem.* **2010**, *58*, 2483−2490.
(46) Hurrell, R. F. Phytic acid degradation as a means of improving iron absorption. *Int. J. Vitam. Nutr. Res.* **2004**, *74*, 445−452.
(47) Ma, G.; Jin, Y.; Plao, J.; Kok, F.; Guusie, B.; Jacobsen, E. Phytate, Calcium, Iron, and Zinc contents and their molar ratios in foods commonly consumed in China. *J. Agric. Food Chem.* **2005**, *53*, 10285−10290.
(48) Hemalatha, S.; Platel, K.; Srinivasan, K. Influence of germination and fermentation on bioaccesibility of zinc and iron from food grains. *Eur. J. Clin. Nutr.* **2007**, *61*, 342−348.